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Evaluation of a recombinant vaccinia virus containing pseudorabies (PR) virus glycoprotein genes gp50, gII, and gIII as a PR vaccine for pigs

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Summary. Pigs vaccinated twice intramuscularly with a highly attenuated strain of vaccinia virus (NYVAC) containing gene inserts for pseudorabies virus (PRV) glycoproteins gp50, gII, and gIII produced neutralizing antibodies for PRV and were less clinically affected than were nonvaccinated pigs following oronasal exposure to virulent PRV. Also, following oronasal exposure to virulent PRV the duration of virulent virus shedding by pigs that had been vaccinated intramuscularly with the recombinant virus was statistically less ($p < 0.05$) than that of nonvaccinated pigs and like that of pigs vaccinated twice intramuscularly with inactivated PR vaccine. Intramuscular vaccination with the recombinant virus was compatible with the most commonly used differential diagnostic tests, namely those based on PRV glycoproteins gX and gI. Serum antibodies for these glycoproteins were absent from the sera of all pigs before and after vaccination with recombinant virus; whereas, they were present in the sera of all of the same pigs after they were exposed to virulent PRV. In contrast to the effectiveness of the recombinant virus administered intramuscularly, neither serum antibody nor clinical protection against PRV was detected when aliquots of the same recombinant virus preparation were administered either orally or intranasally. The latter finding suggests that recombinant virus replicates poorly, if at all, at these sites. If so, the dissemination of recombinant virus from vaccinated pigs to nonvaccinated pigs or other animals in contact seems unlikely.

Introduction

Pseudorabies (PR) is a contagious and sometimes fatal disease caused by a herpesvirus, pseudorabies virus (PRV), of the subfamily *Alphaherpesvirinae* [1]. It affects several species of wild and domesticated animals but is most common in pigs, which also serve as the major interepizootic reservoir and the primary means for dissemination of the causative virus [2–4].

Presently the control of PR of pigs is based largely on the use of vaccines that contain either the entire complement of virus-coded proteins (inactivated and attenuated virus vaccines) [2, 5] or selected viral glycoproteins (subunit vaccine) [6]. All are effective in reducing the clinical effects of the disease. Moreover, attenuated and inactivated vaccines prepared from deletion-mutant strains of PRV, as well as subunit vaccines, can be used in conjunction with appropriate diagnostic tests to identify pigs exposed to virulent, wild-type PRV. Differentiation depends on the detection of antibodies for one or more viral proteins associated only with virulent virus. The most commonly used tests are based on glycoprotein I (gI) [7] or glycoprotein X (gX) [8] which are, respectively, structural and nonstructural glycoproteins of PRV [1]. The primary indication for differential testing is when vaccines are used in conjunction with PR eradication programs (such as those now in progress in the United States and Europe) because, regardless of vaccination history, all pigs that survive exposure to virulent PRV are likely to become latently-infected carriers with the potential for virus reactivation and shedding [9].

Recently another type of vaccine was developd by using a highly attenuated strain (NYVAC) of vaccinia virus as a vector for selected genes of PRV [10]. Three constructs of this vector have already been tested for their immunogenicity in pigs [11]. They differed in that each contained a single gene for one of the three glycoproteins of PRV that are believed to be important immunogens involved in clinical protection, namely, gp50, gII, and gIII. Although the gp50-vectored vaccine appeared to be the most effective, all three stimulated a humoral, virus-neutralizing (VN) antibody response and some degree of clinical protection.

In the study reported here we vaccinated pigs with the NYVAC vector containing PRV genes for gp50, gII, and gIII to test the combined effect of these antigens on immunity. We also compared the effects of administering the recombinant vaccine intramuscularly (IM), intranasally, or orally, and investigated the compatibility of NYVAC-vectored vaccination with two of the differential diagnostic tests (gI and gX) most often used in conjunction with the PR eradication program in the United States.

Materials and methods

Experimental design

Thirty-two 5-week-old pigs were purchased from a commercial herd that was free of infection with PRV. On the day they were delivered to our facility they were weighed, ear tagged, and allocated to five treatment groups (eight pigs for group 1 and six pigs/group for groups 2 through 5), so that each group was comprised of pigs of about the same weight distribution and, thus, the same average weight. Pigs of group 1 were kept as nonvaccinated controls, whereas all pigs of groups 2 through 5 were vaccinated twice at a 28-day interval. Pigs of group 2 were vaccinated (2 ml/dose) IM with inactivated PRV. Pigs of groups 3 through 5 were vaccinated (2 ml/dose) IM (group 3), intranasally (group 4), or orally (group 5) with the NYVAC strain of vaccinia vector containing genes for PRV glycoproteins gp50, gII, and gIII. Infectivity titers and additional details relative to these

vaccines are presented in a subsequent section on "Vaccine preparation." The treatment schedule was: day − 14 to day 0, all pigs were acclimated to our isolation facilities; day 0, first vaccination; day 28, second vaccination; day 56, challenge-exposure, i.e., challenge of immunity by oronasal exposure of each pig to 2 ml of virulent PRV containing 2.8×10^8 plaque forming units (PFU) of virus per ml; day 84, euthanasia by an overdose of barbiturate and necropsy. A blood sample was collected from each pig on days 0, 28, 56, and 84, and the corresponding sera were titered for VN antibodies for PRV. Sera collected on days 0 and 56 were also titered for VN antibodies for vaccinia virus (both the parent NYVAC strain and the recombinant virus). Selected sera also were tested for antibodies for gI and gX. All pigs were weighed at the time they were received on day − 14 and on days 0, 3, 5, 10, 14, 56, 59, 63, 66, 70, and 74. Body (rectal) temperatures were recorded for all pigs on days 0, 3, 5, 10, 14, 56, 57 through 67, 70, and 74. Oropharyngeal swabs were collected from all pigs on days 56, 58, 60, 63, 66, 70, and 74. Swabs were tested for the presence and titer of PRV shedding.

Isolation and observation of pigs

Pigs were kept in four isolation rooms throughout the study. The arrangement from the time they were received until 1 day after they were exposed to virulent PRV (an interval of about 10 weeks, see above) was: room 1, six pigs of group 1 and all pigs of group 2; room 2, two pigs of group 1 and all pigs of group 3; rooms 3 and 4, all pigs of groups 4 and 5, respectively. On the first day after exposure to virulent PRV, two pigs of group 1, room 1, were moved to room 3, and two pigs of group 1, room 1, were moved to room 4, so that each isolation room thereafter contained two pigs of group 1 and all pigs of one other treatment group. All pigs were observed daily throughout the experiment.

Virus neutralizing antibody titrations

Titers of serum VN antibodies for PRV and vaccinia virus were determined by methods previously described [12].

Cell cultures

Bovine embryonic spleen (BESp) cells [13] were used to test oropharyngeal swabs for PRV and a porcine kidney (PK-15) established cell line was used for all other laboratory procedures involving PRV. Primary chicken embryo fibroblasts were used to propagate the NYVAC strain of vaccinia virus for vaccine production and BESp cells were used for all other laboratory procedures involving vaccinia virus. All cells were grown as stationary monolayer cultures in medium that consisted of Eagle's minimal essential medium (EMEM) supplemented with 0.5% lactalbumin hydrolysate, gentamicin sulfate (50 μg/ml) and either 5% fetal bovine serum (FBS) for PK-15 cells or 10% FBS for all other cell types.

Pseudorabies viruses

The attenuated, commercially available, Tolvid strain of PRV containing deletions in its thymidine kinase (TK) and gX genes [14] was used to prepare inactivated PRV vaccine. The virulent Indiana-Funkhauser strain of PRV was used to challenge immunity of pigs and to test sera for VN activity.

Vaccinia virus

The NYVAC strain of vaccinia virus was derived by genetically engineering the deletion of putative virulence and host range genes from the Copenhagen strain of vaccinia virus [10]. The NYVAC strain containing insertions of PRV genes for gp50, gII, and gIII was used to prepare vaccinia-vectored PRV vaccine. The NYVAC strain, both with and without PRV gene insertions, was used to test sera for VN activity.

Vaccine preparation

Inactivated PRV vaccine used in this study was prepared and evaluated previously [9]. The infectivity titer prior to inactivation was 7×10^8 PFU/ml. It was used undiluted except for the addition of 0.1 volume of aluminium hydroxide as an adjuvant.

The vaccinia-vectored PRV vaccine virus was prepared by infecting stationary, monolayer cultures of primary chicken embryo fibroblast at a multiplicity of infection of about 0.1. Two days later, when virus-induced cytopathic effects (CPE) were extensive, the cultures were frozen ($-80°C$) and thawed twice and the cell culture medium was clarified by centrifugation ($500 \times g$, 15 min). The clarified cell culture medium was used undiluted as vaccine. Its infectivity titer was 10^7 median cell culture infective doses ($CCID_{50}$)/ml. Expression of all three PRV glycoproteins by the vaccinia vector was confirmed by propagating an aliquot of the vaccine in BESp and porcine lung cell cultures on coverslips in Leighton tubes and testing replicate, infected cultures by indirect immunofluorescence microscopy against primary sera specific for gII, gIII, or gp50.

Differential diagnostic tests

Selected sera were tested for antibodies for PRV glycoproteins gI and gX using test kits licensed for use in the PR eradication program in the United States (HerdChek: Anti-PRV-gI and HerdChek: Anti-PRV-gpX, IDEXX Laboratories Inc., One IDEXX Drive, Westbrook, ME, U.S.A.). All sera collected from pigs on days 56 and 84 were first tested for antibodies for gI and gX in a regulatory laboratory where such testing is routinely performed (Diagnostic Virology Laboratory, National Veterinary Services Laboratories, USDA, Animal and Plant Health Inspection Service, Ames, IA, U.S.A.). After obtaining the results of these tests we retested all of the same sera plus sera collected from the same pigs on day 0 for the presence of antibodies for gX.

Oropharyngeal swab samples

Procedures for collection of oropharyngeal swab samples, for detection and titration of associated PRV, and for statistical analyses of virus shedding among groups were as previously described [9].

Results

After the first vaccination (days 0 through 28)

All pigs remained clinically normal during the 28 days immediately following the first vaccination. Sera collected from pigs just before the first vaccination (day 0) were free of VN antibody for both PRV and vaccinia virus. Those collected from pigs of groups 1, 3, 4, and 5 at 28 days after the first vaccination also were free of VN antibody for PRV, whereas all of those collected at the same time from pigs of group 2 had VN antibody (Table 1).

Table 1. Virus neutralizing (VN) antibody titers for pseudorabies virus (PRV) in sera obtained from pigs before and after vaccination and challenge exposure

Group	Vaccine	Route of vaccination	Days after 1st vaccination[a]			
			0	28	56	84
1	none	....	<2[b]	<2	<2	9.75
2	inactivated PRV	intramuscular	<2	8.5	9.7	13.3
3	vaccinia vector[c]	intramuscular	<2	<2	7.3	13.3
4	vaccinia vector[c]	oral	<2	<2	<2	10.8
5	vaccinia vector[c]	intranasal	<2	<2	<2	10.3

[a] Swine were vaccinated on days 0 and 28, and exposed to virulent PRV on day 56

[b] Geometric mean titer of VN antibodies

[c] Recombinant vaccinia virus containing PRV glycoprotein genes gp50, gII, and gIII

After the second vaccination (days 28 through 56)

All pigs remained clinically normal during the 28 days immediately following the second vaccination. Sera collected from pigs of groups 1, 4, and 5 just before their immunity was challenged at day 56 were free of VN antibody for PRV. Conversely, sera collected at the same time from pigs of group 3 had VN antibody for PRV, and those collected from pigs of group 2 had an increase in VN antibody for PRV (Table 1). Sera collected from pigs of group 3 on day 56 (i.e. 28 days after the second dose of vaccinia-vectored vaccine) appeared to have a low level of inhibitory activity for vaccinia virus in that the vaccinia-virus-induced CPE usually developed slower at low serum dilutions than it did when the virus was reacted with the same dilutions of prevaccination (day 0) sera from the same pigs. However, the inhibitory activity was never sufficient to block infectivity in all of the cultures even at the lowest dilution (1:2) of serum tested. There was no apparent difference between the reactivity of sera with the NYVAC strain of vaccinia virus either with or without PRV gene insertions.

After challenge of immunity with virulent PRV (days 56 through 84)

All pigs of all treatment groups had clinical signs (inappetence, listlessness, respiratory distress, and in some cases incoordination) following oronasal exposure to virulent PRV. In general, the severity of signs paralleled the increase in body temperatures (Fig. 1). Pigs of groups 2 and 3 appeared less severely affected than did those of groups 1, 4, and 5. One pig of group 4 died 7 days after challenge-exposure, whereas all others recovered. Pigs of groups 2 and 3 gained body weight during the 7 days immediately following challenge-exposure, whereas those of groups 1, 4, and 5 lost weight during the same interval (Fig. 2). All groups had gained weight by the 14th day after challenge-

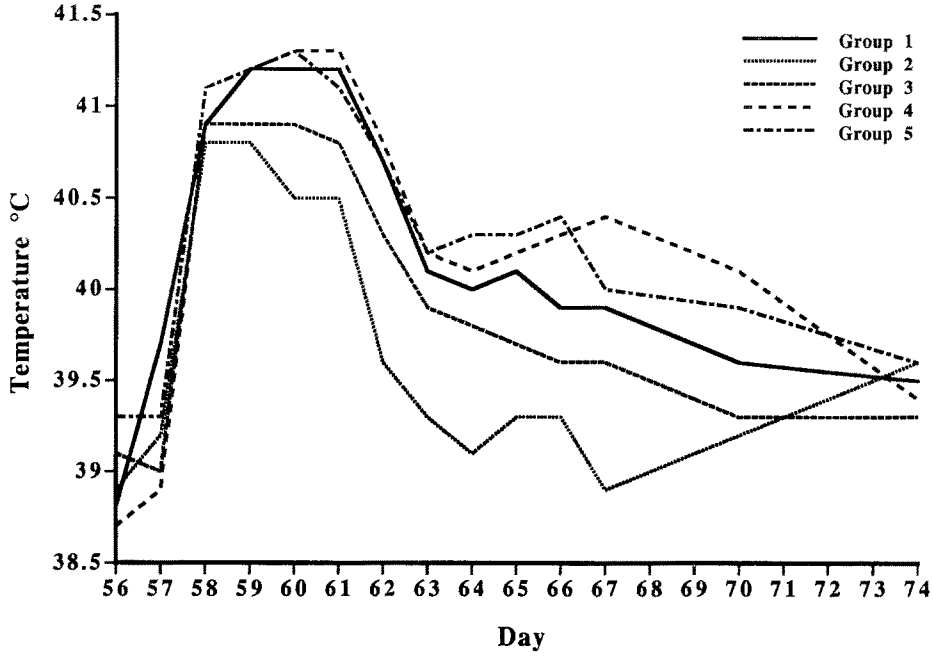

Fig. 1. Mean body (rectal) temperatures of pigs after challenge of immunity by oronasal exposure to virulent pseudorabies virus on day 56

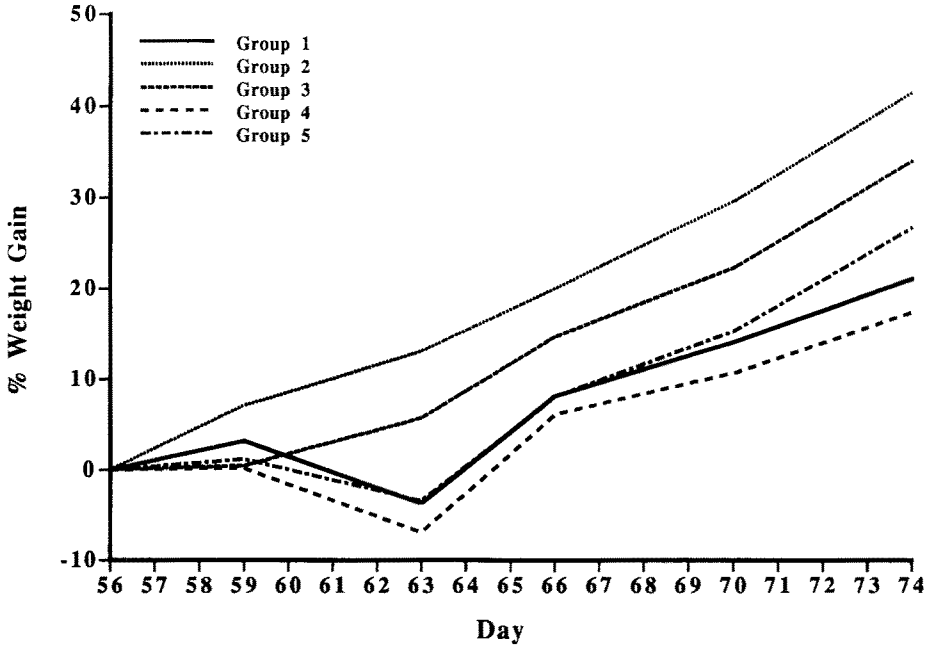

Fig. 2. Mean percent weight gain of pigs after challenge of immunity by oronasal exposure to virulent pseudorabies virus on day 56

Table 2. Duration of shedding of virulent pseudorabies virus (PRV) following challenge exposure

Group	Vaccine	Route of vaccination	Days of virus shedding
1	none	....	3.75 (0.31)[a]
2	inactivated PRV	intramuscular	2.83 (0.31)
3	vaccinia vector[b]	intramuscular	2.33 (0.21)
4	vaccinia vector[b]	oral	3.80 (0.20)
5	vaccinia vector[b]	intranasal	4.17 (0.31)

[a] Mean (standard deviation of the mean); values based on the days oropharyngeal swabs were collected, namely days 56, 58, 60, 63, 66, 70, and 74

[b] Recombinant vaccinia virus containing PRV glycoprotein genes gp50, gII, and gIII

exposure. All pigs had serum VN antibody for PRV on the 28th day after challenge-exposure (day 84); however, the highest titers were in sera of pigs in which antibodies had been raised previously by vaccination (Table 1). All pigs of all groups shed PRV after challenge exposure. For the 6 days on which samples were collected after challenge exposure (i.e., days 58, 60, 63, 66, 70, and 74 of the experiment and days 2, 4, 7, 10, 14, and 18 after challenge) the mean number of days of shedding ranged from 2.3 days for pigs of group 3 to 4.2 days for pigs of group 5 (Table 2). The difference in the duration of shedding between either group 2 or 3 and any of the other groups was statistically significant ($P < 0.05$). On the other hand there was no statistical significance between the duration of shedding of groups 2 and 3.

Differential diagnostic tests

Sera collected on day 56 (just before challenge-exposure) from all pigs of groups 1, 3, 4, and 5 were negative by both the gI and gX differential diagnostic tests. Sera collected at the same time from pigs of group 2 were all positive by the gI differential diagnostic test and two were positive by the gX differential diagnostic test. All of the test results were clearly either positive or negative except for one of the two sera that were positive for antibodies for gX. The reading (optical density) associated with this serum was just low enough for the serum to be classified as positive. The same results were obtained when additional aliquots of the sera collected on day 56 were retested for antibodies for gX, except that the optical density associated with the serum that previously had been just low enough to be classified as positive was, on retest, just sufficient for this serum to be classified as negative, i.e., free of antibodies for gX. Sera collected on day 84 (28 days after challenge-exposure) all contained antibodies for both gX and gI. The same result was obtained when all of these sera were

tested a second time for antibodies for gX. In contrast, none of the sera collected from the same pigs on day 0 (just before the first vaccination) contained antibodies for gX.

Discussion

Pigs vaccinated IM either with inactivated PRV (group 2) or with a vaccinia virus vector containing PRV genes for gII, gIII, and gp50 (group 3) developed immunity to subsequent challenge exposure to virulent PRV. These vaccination procedures appeared to be equally effective in inhibiting shedding of virulent virus after challenge exposure; however, a comparison of temperature responses (Fig. 1) and weight gains (Fig. 2) and our assessment of clinical signs indicated that greater clinical protection was provided by vaccination with inactivated PRV. Whether these differences would be evident in the field where exposure to virulent PRV is likely to be much less than it was in this study, or if they could be affected appreciably by changes in the vaccination procedure, such as by increasing the dose of the vaccinia-vectored vaccine, remains to be determined. Moreover, we emphasize that the inactivated PRV vaccine to which the recombinant was compared may be an exceptionally effective immunogen and perhaps more effective than commercially available inactivated vaccines. During a previous study we found that a single dose of this vaccine stimulated a higher titer of serum VN antibody than did a similar administration of the same strain of attenuated virus [9].

By comparing the results of this study with those of a previous study [11] wherein we tested the same glycoproteins individually, it appears that the combination (gII, gIII, and gp50) is more immunogenic than either gII or gIII alone; however, there is, as yet, no clear evidence from out studies that it is appreciably more immunogenic than gp50 alone. On the basis of serum VN antibody titers and clinical protection against virulent PRV, pigs vaccinated IM with the NYVAC vector containing the gene for gp50 [11] responded at least as well as those vaccinated with the same vector containing all three genes. Because the vaccines were evaluated in different studies, it is possible that other variables affected the results. Moreover, in a study by others [15] in which the same PRV glycoprotein genes were expressed either alone or in combination via a less attenuated vaccinia vector, there was no evidence that gp50 was any more immunogenic than gII or gIII for either mice or pigs that were vaccinated and subsequently exposed to virulent PRV. In fact, mice were protected better by a vector containing gII and gIII than by any of the vector-glycoprotein combinations containing gp50. An explanation for these apparent discrepancies may depend on the relative and/or absolute levels of expression of the different glycoprotein genes by the various recombinants. Nevertheless, if our observations prevail and it is found that the immunity provided by the gp50 vector is sufficient for field use in pigs, other options concerning differential diagnostic tests become obvious, namely, the gp50 vaccine would probably be compatible with differential testing based on other glycoproteins of PRV, such as gIII and

gII. The latter might be particularly useful in that it is both an essential glycoprotein and an excellent antigen which conceivably would always induce a detectable level of homologous serum antibody regardless of the strain of PRV to which a pig had been exposed.

In contrast to the immune response of pigs following IM administration of the vectored vaccine, there was no evidence that either intranasal or oral administration of the same vaccine raised any immunity to PRV (Table 1, Figs. 1 and 2). In this regard the immune response to the vectored PRV vaccine was like that reported previously for inactivated PRV vaccine and unlike the enhanced immunity induced by oronasal administration of live, attenuated PRV [9]. Assuming that the vectored vaccine is infectious for porcine cells in vivo, as we know it to be in vitro, we are left with the possibility that susceptible cell populations are limited or inaccessible by oronasal routes of exposure. If so, the potential for IM-vaccinated swine to disseminate the vector through oronasal secretions to other animals or people in contact would seem to be minimal.

In general, the results of differential testing of sera for antibody to PRV glycoproteins gX and gI were consistent with the histories of exposure through vaccination or challenge or both. Notably, the vaccinia-vectored vaccine appeared to be fully compatible with gX and gI differential tests currently used in the eradication program for PR in the United States. Sera from nonvaccinated pigs and from pigs that were vaccinated with vectored vaccine were free of antibody for both glycoproteins until after their immunity was challenged with virulent (gX+, gI+) PRV. After challenge-exposure, sera from all of the same pigs contained antibody for both glycoproteins. In contrast, all sera from pigs vaccinated with the inactivated Tolvid (gX−, gI+) contained antibodies for gI after vaccination, as well as after challenge exposure. Prevaccination sera of this group were not tested for antibodies to gI; but since all of the other pigs of the experiment were free of antibodies to gI even after vaccination, we can logically assume that seroconversion to gI was due to vaccination. The only unexpected findings of the study in regard to differential testing were the positive test results of gX testing of sera of two pigs that had been vaccinated with inactivated PRV. Because the inactivated vaccine was prepared from a gX deletion mutant, none of the vaccinated pigs should have had post-vaccination antibody to gX unless there is an undeleted portion of the gX gene that codes for the epitope corresponding to the monoclonal antibody used in the test kit. If so, it is not clear why post-vaccination serum from other pigs of group 2 did not also have a clearly positive reaction for antibodies for gX, especially since two doses of the vaccine were administered prior to collection of sera at day 56. Whatever, the fundamental reason(s) for these inconsistencies in gX test results, we assume that they reflect an incompatibility between the test and this particular attenuated vaccine. Previous recognition of the problem is suggested by the fact that the HerdChek: Anti-PRV-gpX differential test is not recommended by its manufacturer for use with Tolvid vaccine.

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